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Molecular-Clinical Correlations in Males With an Expanded FMR1 Mutation

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Fragile X syndrome is caused by an expansion of a CGG repeat in the FMR1 gene. The CGG repeat number of the FMR1 mutation and the percentage of cells with methylation of the gene were studied in 218 male patients. Physical and cognitive measurements were also performed. Patients were divided into three groups; those with full mutation and complete methylation (n = 160), those with full mutation and partial methylation (n = 12), and those with a mosaic pattern (n = 46). Statistical comparisons were made between males with the fully methylated full mutation and those with a mosaic pattern. Males having full mutation with complete methylation had the lowest IQ scores and greatest physical involvement. These significant differences were seen only in ages after puberty. CGG repeat length did not correlate with IQ or the physical index score in any group. These findings suggest that a partial production of FMR1 protein may predict milder clinical involvement in some males with fragile X syndrome. © 1996 Wiley-Liss, Inc.

KEY WORDS: fragile X syndrome, FMR1 gene, FMR protein, methylation, amplification size

INTRODUCTION

As DNA testing in fragile X syndrome (FXS) has become increasingly precise, the exact details of the mutation can now be identified, including CGG repeat number, percentage of methylation, and the presence of full mutation/premutation mosaicism. Normal individuals have approximately 6–52 repeats, premutation carriers have 53–200 repeats and are considered unaffected, and typical fully-affected individuals have >200 repeats and have methylation of the full mutation gene in all cells [Fu et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991; Reiss et al., 1993; Rousseau et al., 1994a]. Some males with the full mutation have only partial methylation (methylation in <100% of cells), and others present with a mosaic pattern: full mutation in some cells, and a premutation in others.

The typical male with full mutation presents with a phenotype that includes mental retardation, prominent ears, long face, and macroorchidism [Hagerman, 1996]. Some connective tissue problems, including hyperextensible joints, pectus excavatum, and flat feet, are also seen in the affected population. Early molecular-clinical studies examined differences between full mutation and premutation carriers, while more recent studies have looked at differences between males with a mosaic pattern and males with full mutation [Rousseau et al., 1991; Staley et al., 1993; Yu et al., 1992]. Although there are clear phenotypic differences between those with a premutation and a full mutation, differences in molecular subtypes of those with a large expansion are more contradictory [de Vries et al., 1993; Hagerman et al., 1994; Loesch et al., 1994; Rousseau et al., 1994a; Staley et al., 1993].

Staley et al. [1993] showed that males with full mutation have lower IQs than mosaic males. Other reports have supported the finding of higher-functioning mosaic males [Heitz et al., 1992; Knight et al., 1992; Verheij et al., 1993; Willems et al., 1992]. However, several large studies did not find a significant difference in the cognitive ability of full-mutation males compared to mosaic males [de Vries et al., 1993; Rousseau et al., 1991]. Some of these studies documented a correlation between phenotypic involvement (including physical and cognitive traits) and length of CGG expansion within the full mutation range in males [Loesch et al., 1993: Rousseau et al., 1994a; Yu et al., 1992]. Baumgardner et al. [1995] focused on the neurobehavioral phenotype of males and did not find any correlation between CGG amplification and phenotypic involvement. These researchers also did not find any phenotypic differences between patients with mosaicism and those with full mutation. However, Verheij et al. [1993] reported a limited level of fragile X mental retardation 1

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protein (FMRP) production in a male with a mosaic pattern. Loesch et al. [1993] found differences in both cognitive and physical measures within the full mutation range, depending on length of CGG repeat. Mildly-affected individuals had a CGG repeat at the low end of the full mutation range. Thus, perhaps, a significant level of FMRP is produced in some mosaic males or males with a CGG expansion at the low end of the full mutation range, which could explain the milder physical or cognitive involvement in these males.

Several recent reports have focused on males with methylation variations (full mutations with less than complete methylation), and have shown that these individuals have better cognitive skills when compared to fully-methylated, full-mutation individuals. Rousseau et al. [1994b] documented a case of a mentally normal male with full mutation and only 40% methylation. Hagerman et al. [1994] and Merenstein et al. [1994] found a total of 4 males who maintained an IQ in the nonretarded range (>70) through adolescence or adulthood, and had almost completely unmethylated full mutations. Western blot studies demonstrated that lymphoblastoid cell lines from 2 of these high-functioning males were producing approximately 35% of normal levels of FMRP. Merenstein et al. [1994] detailed a case study of one of these high-functioning individuals who maintained a normal full-scale IQ into adulthood, but had significant deficits in social and emotional aspects of daily life. This patient produced 10% of the normal level of FMRP. Loesch et al. [1993] showed higher cognitive abilities in 3 males with a lack of methylation of a full mutation, and McConkie-Rosell et al. [1993] reported mild phenotypic involvement in 2 brothers with full mutations that were only 3% methylated. Devys et al. [1993] also reported FMRP production in a male whose full mutation was partially unmethylated. This individual had cognitive deficits. In the largest previous study of males with partial methylation (21 patients), Taylor et al. [1994] documented that all males with <10% methylation had IQs in the nonretarded range and produced FMRP at levels between 20–60% of normal, while males with >50% methylation of their full mutation produced very little FMRP.

All of these data suggest a spectrum of involvement in individuals, depending on the details of the molecular findings. The ultimate effect of the molecular findings (i.e., CGG repeat level, methylation status, or the presence of mosaicism) is on the amount of FMRP produced in a particular individual. Absence of FMRP causes typical fragile X syndrome, including moderate-to-severe mental retardation, hyperactivity, anxiety, social avoidance, and mood lability, sometimes with aggression. Detectable levels of FMRP may result in higher cognitive skills, but still may not be sufficient to prevent problems related to emotion and behavior. This article is a further exploration of the spectrum of involvement of fragile X syndrome by elucidating the physical, behavioral, and cognitive differences in males with full mutation with complete methylation, full mutation with partial methylation, and full mutation/premutation mosaicism. We hypothesize that some patients with mosaicism or partial methylation produce a limited level of FMRP, and that the higher the level of FMRP production, the lower the degree of fragile X syndrome phenotypic involvement. Therefore, phenotypic differences should be present in some males with a mosaic or partial methylation pattern, compared to males with a fully-methylated full mutation.

MATERIALS AND METHODS

Physical, cognitive, behavioral, and molecular genetic data were collected on 218 males with fragile X syndrome and an expanded repeat number >200. Of those 218, 125 (59%) were prepubertal (age 3 months–12 years), and 93 (41%) were >12 years old (which we labelled postpubertal). Blood samples for DNA testing were obtained from patients with the mutation, who are currently being followed in the Child Development Unit at the Children's Hospital (Denver, CO). Subjects ranged from age 3 months–60 years, with a mean age of 12.9 years (Table I and II).

Of the 218 subjects, 119 underwent psychological testing (37% of the prepubertal males, and 84% of the postpubertal group), and full-scale IQ scores were obtained from one of the following age-appropriate batteries: Wechsler Adult Intelligence Scales—Revised, Wechsler Intelligence Scale for Children—Revised (WISC-R) and WISC III, Stanford-Binet, or the Kaufman Assessment Battery for Children. A development quotient (DQ) was obtained in children under age 3 years by utilizing the Bayley Scales of Infant Development.

Subjects were given a detailed medical examination and participated in a medical and behavioral history-taking session. Parents or caretakers were asked about the presence of a variety of behaviors, such as handbiting and handflapping (listed in Table III). The frequency of these behaviors was not assessed in each individual, because they typically vary from day to day. The tables show the incidence of these behaviors as they were documented within each patient age group. Physical examination included height and weight measurements and anthropometric measures of the head and neck area, including: inner and outer canthal distances, length of palpebral fissure, occipital frontal circumference, head and face length, ear length and width, and inner and outer ear prominence measurements, as previously described in Hull and Hagerman [1993]. The subjects were given a physical index score (PI) score based on the presence or absence of 10 of the most common physical traits found in fragile X syndrome [Cronister et al., 1991; Hagerman, 1996]. These include long face, long ears (>7.0 cm), prominent ears, high-arched palate, flat feet, hyperextensible metacarpal phalangeal joints (extension >90°), double-jointed thumbs, heart murmur or click, Sydney or simian palmar crease, and hand calluses. For each trait present, 1 point was given, so that the PI score ranged from 0–10 in each patient, as previously described [Cronister et al., 1991]. This PI score has been shown to statistically differentiate individuals with full mutation from controls, and even individuals with premutations from controls [Hull and Hagerman, 1993]. In addition, males were assessed for macroorchidism. All subjects were examined by the same physician, who was unaware of the details of the molecular findings at the time of physical examination

(R.J.H.). Table II shows the range and means for data in the cognitive field, and for selected physical measurements yielding a PI score and percentile height.

DNA diagnostic analysis was performed by direct probing of genomic DNA to determine FMR1 mutation and CGG repeat number status of the 218 patients studied. In addition, methylation status at the FMR1 CpG *island was analyzed* in 211 patients. Over several years, molecular diagnostic testing was performed at three centers: Kimball Genetics in Denver, using a Southern blot assay with FMR1 probe StB 12.3 [Rousseau et al., 1991] and a PCR-based assay using primers 1 and 3, as previously described [Brown et al., 1993]; the Children's Hospital in Denver, using a Southern blot assay with the probe Oxl. 9 [Bell et al., 1991] and a PCR-based assay [Pergolizzi et al., 1992]; and at the University of Colorado Health Science Center DNA Diagnostic Laboratory, using a Southern blot assay with FMR1 probe StB 12.3 and a PCR-based assay. The subjects were then categorized based on variations of the mutation: group 1 had full mutations with full methylation (FMFM); group 2 had full mutations with partial (<50%) methylation (FMPM); and group 3 had mosaicism, with a premutation in some cells and full mutation in others. (Table I). The partial-methylation group was selected to have <50% methylation, because data of Taylor et al. [1994] demonstrated that this subgroup is most likely to produce FMRP. Percentage of methylation was determined by phosphoroimaging to measure Southern blot band intensity, and by subsequent calculation of percentage of contribution of the methylated full-mutation signal to the total methylated and unmethylated full-mutation signal.

RESULTS

Of primary interest was whether the cognitive, behavioral, and physical scores from each of the three groups (full mutation/full methylation (FMFM), full mutation/partial methylation (FMPM), and mosaic) would show significant differences. The dependent variables included: full-scale IQ; physical index score (PI); anthropometric measurements of the head and neck; the 10 physical signs making up the PI score; macroorchidism; pectus excavatum; the centile height, weight, and head circumference; and behavior, including presence of hand flapping, hand biting, hyperactivity, aggression, violent outbursts, shyness, anxiety, and tactile defensiveness. Age and height were examined within both prepubertal and pubertal age ranges. The means were not significantly different within each group (Table I).

Since the numbers were too small in the FMPM group to include in a statistical analysis, only the FMFM males were compared to males with a mosaic pattern. Student's t-test was performed to examine statistical information between the two groups. Table II shows statistical significance for the postpubertal age range, with FMFM males having lower FSIQs ($P = .00001$) and a higher overall PI score ($P = .01$). No significant differences were found between groups in the prepubertal age range.

Tables III and IV give the information for all three groups, using frequency data from acquired behavioral and physical information. There were no significant differences in the prepubertal physical or behavioral data. In the postpubertal age range, significant differences in behavior patterns were seen specifically for shyness ($P = .01$). In physical measurements, FMFM males had a higher incidence of prominent ears ($P = .02$), a higher incidence of high-arched palate ($P = .048$), and a lower incidence of single palmar crease ($P = .03$) compared to males with a mosaic pattern.

CGG Amplification

A bivariate correlation was performed to examine if length of CGG repeat correlated with any aspect of the typical phenotype. This correlation was carried out for individuals who had an exact CGG repeat range documented in the fully-methylated group (n = 53) and in the mosaic group (n = 27). Too few individuals were available for this analysis in the partially-methylated group (n = 4). The lowest band-size of the CGG expansion in the full-mutation range was used as previously described and utilized by Abrams et al. [1994]. This correlation was carried out with several continuous variables, including: full-scale IQ, Connors scale [1973] for rating hyperactivity, PI score, outer ear prominence, ear width, ear length, head height. The correlation was also run with variables indicating presence or lack of a trait, including the traits making up the PI score, and selected behavioral data, including hand flapping, hand biting, hyperactivity, poor eye contact, and perseveration. No correlation was found between CGG repeat length and any phenotypic data, except for single palmar creases in the full-mutation/full-methylation group ($r = -.42$; $P = .002$). Given the large number of analyses, we would expect at least one correlation by chance at this level, and this correlation is not clinically mean-

TABLE I. Group Characteristics

Prepubertal group	Description	Mean age in years (SD)	Mean height in inches (SD)
Group 1 (n = 96)	Full mutation, full methylation	6.4 (3.0)	46.3 (9.7)
Group 2 (n = 5)	Full mutation, <50% methylation	6.4 (3.0)	46.4 (9.1)
Group 3 (n = 24)	Mosaic	6.9 (3.1)	47.3 (8.1)
Postpubertal group	**Description**	**Mean age in years (SD)**	**Mean height in inches (SD)**
Group 1 (n = 64)	Full mutation, full methylation	22.4 (8.7)	66.8 (4.8)
Group 2 (n = 7)	Full mutation, <50% methylation	22.1 (7.5)	71.1 (3.1)
Group 3 (n = 22)	Mosaic	22.6 (12.2)	66.8 (5.4)

TABLE II. IQ, Physical Index Scores, and Percentile Height for Each Mutation Category

Prepubertal group	(n)	Mean (SD)	Range of measures
		FSIQ	
1 FMFM	35	51.3 (11.4)	30–80
2 FMPM	2	63.5 (4.9)	60–67
3 Mosaic	10	65.6 (19.4)	37–89
		Physical index score	
1 FMFM	91	4.8 (1.6)	1–10
2 FMPM	5	3.8 (1.3)	2–5
3 Mosaic	24	4.7 (1.6)	2–8
		Centile height	
1 FMFM	93	50.2 (30.9)	5–98
2 FMPM	5	48.0 (24.9)	30–90
3 Mosaic	24	56.8 (29.0)	5–95
Postpubertal group			
		FSIQ*	
1 FMFM	51	41.2 (11.8)	15–66
2 FMPM	4	88.2 (11.7)	73–100
3 Mosaic	17	60.1 (15.0)	40–92
		Physical index score**	
1 FMFM	60	5.1 (1.8)	0–10
2 FMPM	6	3.8 (1.4)	2–6
3 Mosaic	21	3.9 (1.5)	0–6
		Centile height	
1 FMFM	56	47.2 (3.4)	5–93
2 FMPM	6	87.2 (3.4)	80–98
3 Mosaic	19	54.4 (5.7)	5–90

*F (2, 69) = 35.59, P = .00001.
**F (2, 84) = 4.36, P = .01.

TABLE III. Group Behavior Information†

	Prepubertal group[a]		
Category	Group 1, FMFM (n = 96)	Group 2, FMPM (n = 5)	Group 3, Mosaic (n = 24)
Hand flapping	82 (85%)	4 (80%)	21 (89%)
Hand biting	61 (64%)	2 (40%)	10 (42%)
Hyperactive	85 (89%)	3 (60%)	23 (96%)
Perseverate	91 (95%)	3 (60%)	22 (95%)
Imitate	75 (78%)	NA	20 (85%)
Aggression	55 (57%)	3 (60%)	16 (65%)
Shyness	58 (60%)	4 (75%)	11 (44%)
Anxiety	61 (64%)	4 (75%)	13 (53%)
Panic attacks	24 (25%)	3 (50%)	6 (25%)
Poor eye contact	84 (88%)	4 (80%)	21 (87%)
Violent outbursts	24 (25%)	1 (25%)	7 (28%)
Tactile defensiveness	73 (76%)	1 (25%)	18 (73%)
	Postpubertal group[a]		
Category	Group 1, FMFM (n = 64)	Group 2, FMPM (n = 7)	Group 3, Mosaic (n = 22)
Hand flapping	52 (81%)	1 (17%)	15 (68%)
Hand biting	41 (64%)	1 (17%)	11 (50%)
Hyperactive	41 (64%)	5 (67%)	18 (82%)
Perseverate	64 (100%)	5 (75%)	20 (91%)
Imitate	53 (83%)	4 (60%)	19 (88%)
Aggression	35 (55%)	5 (67%)	10 (47%)
Shyness*	39 (61%)	7 (100%)	22 (100%)
Anxiety	51 (79%)	7 (100%)	20 (91%)
Panic attacks	25 (39%)	2 (25%)	5 (22%)
Poor eye contact	63 (98%)	5 (67%)	22 (100%)
Violent outbursts	27 (42%)	2 (20%)	8 (37%)
Tactile defensiveness	55 (86%)	4 (60%)	18 (80%)

†Statistical analysis applies to groups 1 and 3 only.
[a]Number of individuals in each group who were positive for these phenotypic characteristics.
*F(1, 41) = 7.23, P = .01.

TABLE IV. Group Physical Information†

Category	Prepubertal group[a]		
	Group 1, FMFM (n = 96)	Group 2, FMPM (n = 5)	Group 3, Mosaic (n = 24)
Long ears	24 (25%)	1 (25%)	5 (21%)
Long face	61 (64%)	2 (40%)	15 (63%)
Prominent ears	75 (78%)	1 (20%)	21 (86%)
High-arched palate	49 (51%)	5 (100%)	9 (36%)
Hyperextensible joints	78 (81%)	4 (80%)	21 (86%)
Double-jointed thumb	56 (58%)	4 (80%)	15 (62%)
Palmar crease	25 (26%)	2 (40%)	4 (14%)
Hand calluses	17 (18%)	1 (20%)	3 (13%)
Flat feet	79 (82%)	4 (80%)	20 (82%)
Heart murmur/click	15 (16%)	0 (0%)	2 (7%)
Macroorchidism	52 (54%)	2 (40%)	13 (54%)
	Postpubertal group[a]		
Category	Group 1, FMFM (n = 64)	Group 2, FMPM (n = 7)	Group 3, mosaic (n = 22)
Long ears	32 (50%)	4 (50%)	6 (27%)
Long face	51 (80%)	6 (83%)	16 (71%)
Prominent ears*	42 (66%)	NA	8 (36%)
High-arched palate**	40 (63%)	NA	9 (38%)
Hyperextensible joints	31 (49%)	1 (17%)	11 (50%)
Double-jointed thumb	30 (48%)	1 (17%)	8 (35%)
Palmar crease***	14 (22%)	0 (0%)	10 (48%)
Hand calluses	33 (52%)	0 (0%)	9 (40%)
Flat feet	38 (60%)	1 (17%)	11 (50%)
Heart murmur/click	19 (29%)	2 (33%)	2 (9%)
Macroorchidism	59 (92%)	6 (83%)	20 (91%)

†Statistical analysis applies to groups 1 and 3 only.
[a]Number of individuals in each group who were positive for these phenotypic characteristics.
*$F(1, 81) = 5.92, P = .02$.
**$F(1, 81) = 4.03, P = .048$.
***$F(1, 76) = 4.70, P = .03$.

ingful (i.e., the larger the CGG repeat length, the less likely individuals were to have a single palmar crease).

DISCUSSION

This study has documented less phenotypic involvement in males who have a mosaic pattern compared to males with the full mutation. Differences exist not only in full-scale IQ, but also in overall physical index score, shyness, and some individual physical findings which have not been previously studied. It is interesting that all of these differences do not become clear until adulthood. For instance, IQ becomes significantly higher only in adulthood between mosaic males and fully-methylated males. This is probably related to the fact that a significant number of preschool males with fragile X syndrome have a nonretarded IQ [Freund et al., 1995], but subsequent significant IQ decline occurs throughout childhood. This IQ decline may be less pronounced in those individuals who can produce at least a limited amount of FMRP, which is the case for some males with a mosaic pattern [Verheij et al., 1993] and some males with an unmethylated or partially-methylated full mutation [Hagerman et al., 1994; Merenstein et al., 1994; Taylor et al., 1994]. Although the numbers were too small for a statistical analysis, males with a partially-methylated full mutation appear to be the tallest and to have the highest IQ of all the males with fragile X syndrome. These findings support the notion that FMRP continues to be important in brain development throughout childhood [Reiss et al., 1994], influencing cognitive, behavioral, and even growth in fragile X syndrome [Loesch et al., 1995].

An interesting finding was the higher incidence of shyness in adulthood in mosaic males compared to males with complete methylation. This suggests that shyness is a greater problem in mildly affected individuals than in more severely affected males. This is supported by data on females with the full mutation who are usually shy even when their cognitive abilities are in the normal range [Freund et al., 1993; Hagerman et al., 1992]. In addition, Sobesky et al. [1995] have shown that greater hyperactivity in girls appears to be protective of severe shyness. Hyperactivity is associated with impulsivity and social outgoingness, so shyness is less of a problem in individuals who are hyperactive. Individuals who are more significantly affected by fragile X syndrome have more frequent hyperactivity. In addition, significantly less frequent shyness was seen in adults with complete methylation compared to mosaic adults, suggesting that hyperactivity may counteract problems with shyness, as in females. Hyperactivity is related to frontal or executive function deficits, and this effect is seen early on in individuals, including females, affected by fragile X syndrome [Mazzocco et al., 1993].

We were not surprised by the lack of correlation between CGG repeat number and phenotypic measures in males with full methylation and mosaic males. Although others have reported a limited association

[Loesch et al., 1993; Rousseau et al., 1994a], previous reports did not remove individuals who were only partially methylated, as we did. The association in previous reports is probably due to CGG repeat numbers at the low end of the full mutation range that may have been unmethylated and transcribing some FMR1 message in some tissues. However, Feng et al. [1995] have shown for unmethylated mutations that transcription takes place, but that translation is severely inhibited when the message has >230 repeats. Once the CGG repeat number is large enough to be methylated in all cells and completely shuts down transcription and translation, there is no FMR1 protein produced, and differences in CGG repeat number should not make a significant difference in phenotypic involvement.

Further FMR1 protein studies using immunohistochemistry [Willemsen et al., 1995] or ELISA assays must be carried out to gain a better understanding of the improved prognosis of males who have partial methylation and of the subgroup of mosaic males who may show significant FMRP production.

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